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Antioxidant and anti-inflammatory functionality of ten Sri Lankan seaweed extracts obtained by carbohydrase assisted extraction

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Abstract Enzyme-assisted extraction is a cost-effective, safe, and efficient method to obtain bioactives from plant materials. During this study, 10 different marine algae from Sri Lanka were individually extracted by using five commercial food-grade carbohydrases. The enzymatic and water extracts of the seaweeds were analyzed for their antioxidant and anti-inflammatory activities. The highest DPPH, hydrogen peroxide (H_2O_2) and intracellular H_2O_2 scavenging abilities were observed from the Celluclast extract of *Sargassum polycystum* (CSp). CSp exerted protective effects against oxidative stress-induced cell death in hydrogen peroxide-induced Chang cells and in model zebrafish. The Celluclast extract of *Chnoospora minima* (CCm) showed the strongest anti-inflammatory activity against lipopolysaccharide (LPS)-induced NO production in RAW 264.7 macrophages (IC_{50} = 44.47 μg/mL) and in model zebrafish. CCm inhibited the levels of iNOS, COX-2, PGE_2, and TNF-α in LPS stimulated RAW 264.7 macrophages. Hence, CSp and CCm could be utilized in developing functional ingredients for foods, and cosmeceuticals.

Keywords Enzyme-assistant extraction · Celluclast · Marine algae · *Sargassum polycystum* · *Chnoospora minima* · Antioxidant

Introduction

Studies centered on the evaluation of natural products from marine algae have garnered much attention from the food, cosmetic and pharmaceutical industries over the last few years. A range of biologically active secondary metabolites including polyphenols, terpenoids, sulfated polysaccharides, alkaloids, polyunsaturated fatty acids, mycosporine-like amino acids, peptides and halogenated compounds have been identified from marine algae (Bhakuni and Rawat, 2006). These molecules are active against many diseases. Unlike most of the natural products isolated from terrestrial organisms, these molecules possess unique structural properties, which have extended the research perspectives from characterization to the synthesis of structural analogs (Li and Vederas, 2009). A typical method employed in the extraction of natural products is the use of organic solvents. However, new environmental-friendly extraction techniques are required for natural product research and potential industrial applications. Enzyme-assisted extraction (EAE) is an innovative technique that enables a higher extraction efficiency to be obtained. The enzymes assist in breaking the cellular matrix and cell walls, allowing the release of compounds that stay attached to the cell walls and inside the cytosol (Wijesinghe and Jeon, 2012). Hydrolytic enzymes that specifically degrade polysaccharides and proteins can be the key to producing new macromolecular substructures with potentially beneficial functionalities. In particular, marine algae contain sulfated polysaccharides, with a wide

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range of biological activities, which have not been reported in terrestrial plants. Recently, the enzymatic hydrolysis of sulfated algal polysaccharides has attracted special attention (Hehemann et al., 2010). EAE of algae has also been implemented for the extraction of natural products with antioxidant, anti-inflammatory, anticoagulant and antiproliferative effects (Athukorala et al., 2006; Lee et al., 2011; Samarakoon et al., 2013b).

The investigation of antioxidant effects is central to the fields of natural product research and food research, as these compounds are beneficial for physiological wellbeing, to counteract oxidative stress, and to mitigate the pathogenesis of disease conditions, such as rheumatoid arthritis, cancer, atherosclerosis, and cardiovascular and neurodegenerative diseases (Pham-Huy et al., 2008). Alternatively, these compounds can increase the shelf life of food through the reduction of lipid peroxidation. Phenolic compounds are noted for their abundance in marine algae (Goad, 1978). Anti-inflammatory activity is another interesting area of research, as inflammatory diseases have emerged as a prominent health issue in many parts of the world, exerting a considerable influence on healthcare costs. Inflammation is part of the complex stereotypical responses of the body to harmful stimuli; these inflammatory responses are mediated through a complex system of cell signaling pathways, which involves cytokines and lipid mediators (Fernando et al., 2016b). Inflammatory responses are crucial to allow an organism to counteract infection. However, chronic inflammation can result in detrimental issues to physiological well-being owing to an array of degenerative disease conditions, including inflammatory arthritis, coronary artery diseases, multiple sclerosis, cancer, obesity, atherosclerosis, migraines, interstitial cystitis, dermatitis, insulin resistance, and irritable bowel syndrome (Coussens and Werb, 2002; Hansson, 2005; Xu et al., 2003).

According to Coppejans et al. (2009), the coastal areas of Sri Lanka offer suitable habitats for the growth and distribution of a wide diversity of algae species. However, except for a few studies, the bioactive properties of components from these organisms are unexplored (Fernando et al., 2017a; 2017b; Lakmal et al., 2014; Mahendran et al., 1980; Premakumara et al., 1996). Fernando et al., (2017b) described the identification of antioxidant activities of water-soluble polysaccharides from several algae species. In another study, Fernando et al. (2017a) described the purification and anti-inflammatory effects of a fucoidan from *Chnoospora minima*. Mahendran et al. (1980) reported the identification of sterols from 18 Sri Lankan algae by using GC–MS. According to Lakmal et al. (2014) promising anticancer and antioxidant properties of Sri Lankan algae were observed from the methanol extracts of *C. racemosa*. Premakumara et al. (1996) reported the isolation of a non-steroidal contragestative agent from *Gelidiella acerosa*. The present study aimed to explore the biofunctional properties of the enzymatic extracts of Sri Lankan marine algae in order to examine their possible incorporation in functional food or industrial applications.

Materials and methods

Viscozyme L, Celluclast 1.5L FG, AMG 300L (an exo 1,4-alpha-d-glucosidase), Termamyl 120L and Ultraflo L were obtained from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). Chang liver cells and RAW 264.7 macrophages were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) was purchased from GIBCO Inc. (New York, NY, USA). Phenylthiourea (PTU), 2′,7′-2′ 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), acridine orange, ethidium bromide, Hoechst 33342 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma, Aldrich, USA. All the other chemicals used in this study were of high purity grade.

Sample collection and preparation

The algae were collected from the southern, northwestern and northeastern locations of Sri Lanka during May 2015. Samples were identified according to its morphological and anatomical characteristics referring to the guides which provide detailed taxonomical information about Sri Lankan algae (Coppejans et al., 2009; Durairatnam, 1961). *Sargassum polycystum*, *Sargassum natans*, *Padina commersonii*, *Ahnfeltiopsis pygmaea* and *Gracilaria corticata* were collected from the Hikkaduwa area, *Cladophora herpestica Chaetomorpha antennina* and *Grateloupia lithophila* from the Kalpitiya area while *Chnoospora minima* and *Ulva fasciata* from the Nilaveli area. All the samples were thoroughly washed using tap water to remove sand, salt, and attached epiphytes. Samples were then freeze-dried and ground to a powder.

Enzyme-assistant extraction (EAE)

Two grams of the cryodesiccated powdered algae was seperatelly suspended in 100 mL of deionized water and adjusted to the optimum pH values of the enzymes using either 1 M HCl or NaOH. Enzymes were separately introduced into each sample suspension at a concentration of 0.5% and were incubated at their optimum temperature for 24 h under continuous agitation. The optimum conditions are as follows; Viscozyme (50 °C, pH 4.5), Celluclast (50 °C, pH 4.5), AMG (60 °C, pH 4.5), Termamyl (60 °C,

pH 6.0) and Ultraflo (0 °C, pH 7.0) (Wijesinghe and Jeon, 2012). The enzymes were inactivated by heating in a boiling water bath for 10 min. Finally, the hydrolysates were filtered, and the pH was re-adjusted to 7.0. In addition to EAE, each 2 g of powdered algae was extracted by using deionized water at 30 °C. Finally, all the extracts were lyophilized.

Preliminary analysis

The algae were analyzed for their proximate chemical composition according to AOAC 2005 standards (Association Of Official Analytical, 1998). The protein and the lipid content of the algae were determined using Kjeldahl and Soxhlet methods, respectively. The ash content was analyzed by dry ashing in a furnace at 550 °C for 6 h. The polysaccharide contents were determined by phenol–sulfuric acid method (DuBois et al., 1956). Each extract was analyzed for its yield, total polyphenolic content by Folin–Ciocalteu method (Chandler and Dodds, 1983), carbohydrate content by phenol–sulfuric acid method (DuBois et al., 1956) and protein content using Thermo scientific Pierce BCA protein assay kit. The mineral composition of algae was analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES) (PerkinElmer® Optima 7300 New York, NY, USA) following acid digestion (HCl/HNO_3 3:1) as described by Pasławski and Migaszewski (Pasławski and Migaszewski, 2006).

Evaluation of antioxidant activities

Antioxidant activities of the extracts were analyzed each at 2 mg/mL sample concentration. Distilled water instead of sample extracts was used as the control in each colorimetric assay while maintaining necessary blanks for each sample. DPPH radical scavenging activity was measured according to Yang et al. (2011). The absorbance was measured at 517 nm using a synergy HT multi-detection microplate reader (BioTeek, Winooski, VT, USA). The hydrogen peroxide scavenging activity was determined according to the method by Siriwardhana et al. (2004). The ferrous ion chelating ability was measured according to the method described by Lee et al. (2009). Percentage antioxidant activities were calculated using the following equation:

$$\text{Anti-oxidant activity } (\%) = [(A_0 - (A_1 - A_2))]/ A_0 \times 100$$

where A_0 indicates the absorbance of the control, A_1 indicates the absorbance of the sample and A_2 indicates the absorbance of the respective blank.

Cell culture

Cytotoxicity and intracellular ROS scavenging effects of the extracts were determined using Chang cells derived from the human liver tissue. The anti-inflammatory activity was evaluated using RAW 264.7 macrophages. Both cell lines were maintained in DMEM medium supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). Cells were stored at 37 °C in a humidified atmosphere with 5% CO_2. Cells under an exponential growth were seeded for the experiments. MTT colorimetric assay was employed for the determination of cell viability as described by Samarakoon et al. (2013a).

Intracellular ROS scavenging activity

Intracellular ROS scavenging ability of the extracts was measured using dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay as described by Yang et al. (2011). Samples were treated at 100, 50 and 25 mg/mL concentrations to a 96 well plate seeded with Chang cells (1×10^5 cells/mL) and treated with H_2O_2 (1 mM). After 1 h incubation, 10 µl of DCFH-DA (25 µg/mL) was treated into each well. Fluorescence was measured using a synergy HT multi-detection microplate reader (BioTeek, Winooski, VT, USA) at an excitation and an emission wavelength of 485 and 530 nm, respectively following a 10 min incubation.

Lipopolysaccharide (LPS)-induced NO production and cytotoxicity of RAW 264.7 macrophages

Experiments were carried out according to the method by Ko and Jeon (2015) on RAW 264.7 macrophages. Samples were treated at 200, 100 and 50 µg/mL concentrations. NO production was measured by Griess assay, and cell viability was evaluated using MTT assay.

Western blot analysis

Experiments were performed according to Lee et al. (2015). RAW 264.7 cells were treated with 25, 50 and 100 µg/mL of the Celluclast extract of *C. minima* (CCm). Cells were harvested using PBS, and the cell lysates were prepared using lysis buffer. Cellular proteins were collected by centrifugation (12,000 rpm) and analyzed using Thermo scientific Pierce BCA protein assay kit. Lysates were standardized and subjected to SDS-PAGE. The resolved protein bands were transferred onto polyvinylidene fluoride membranes (Bio-Rad, Irvine, CA, USA). Nonspecific binding sites on the membranes were blocked by blocking solution containing 5% nonfat dry milk in Tris-buffered saline and Tween-20. Membranes were then

incubated sequentially with primary and secondary antibodies. Westar EtaC enhanced chemiluminescent substrate (Cyanagen Srl, Bologna, Italy) was used to develop the western blot signals and the image analysis was done using a fluorescence molecular imaging system (Vilber Lourmat, Paris, France).

In vivo evaluation of intracellular hydrogen peroxide scavenging and anti-inflammatory activities using zebrafish embryos

Zebrafish embryos were obtained by natural spawning (Kang et al., 2013). At 3 h post-fertilization (hpf), embryos (15/group) were transferred to 12-well plates and mounted in embryo media. After 1 h, the wells were treated with 50, 100 and 200 μg/mL of Celluclast extract of *S. polycystum* (CSp) for evaluating intracellular hydrogen peroxide scavenging activity and with 25, 50 and 100 μg/mL of CCm for evaluating anti-inflammatory activity. After 1 h incubation, H_2O_2 (10 μg/mL) or LPS (10 μg/mL) was introduced into the respective wells of the plates. After 24 h the embryos were rinsed and mounted in fresh embryo media containing 0.2 mM PTU. For evaluating viability factors experimental fish embryos and the hatched larvae were daily monitored for survival and heartbeat rate up to 5 days.

For the evaluation of ROS, NO levels, and cell death, at 3 day postfertilization (dpf) zebrafish larvae were transferred to 12 well plates and treated with different fluorescent probe dyes (Kang et al., 2013). ROS levels were detected using DCFH-DA (20 μg/mL). NO levels were detected using DAF-FM-DA solution (5 μM), and the cell death was detected using acridine orange staining (7 μg/mL). Photographs were taken using a microscopic CoolSNAP-Pro color digital camera (Olympus, Japan). The fluorescence intensities were quantified using image J program.

Statistical analysis

All the data values are expressed as the mean ± standard deviation (SD) based on at least three independent experiments. Statistical analysis for comparing the data was performed using IBM SPSS Statistics 20 software using one-way ANOVA followed by Duncan's multiple range test (DMRT). P values less than 0.05 ($P < 0.05$) were considered as significant.

Results and discussion

Proximate composition

The proximate ash, protein, carbohydrate and lipid composition of the ten algae were estimated in accordance with the standard methods described in AOAC 2005. The results are provided in the Supplementary Materials (Supplementary Table 1). The highest protein and carbohydrate content was observed in *U. fasciata* and *G. lithophila,* respectively. The ash content was higher in *C. herpestica* and *P. commersonii*, which are predominantly composed of Ca^{2+} ions (Supplementary Table 2). These observations were related to their calcified morphology. High amounts of K, Ca, Na, and Mg were detected in all samples. Considerable levels of Arsenic (As) were detected in some algae; however, the values were within the normal levels encountered in algae (Rose et al., 2007).

Extraction yields, chemical composition, and antioxidant activities

The EAE of algae resulted in a higher yield than the water extraction process (Supplementary Table 3). In addition, the content of carbohydrates, proteins, and polyphenols, were lower after water extraction than after the EAE, which suggested that carbohydrases were more effective in yielding the above organic compounds as a result of breakage of cell walls. As given in Supplementary Table 3, the Celluclast extract of *S. polycystum* (CSp) at 2 mg/mL contained the strongest DPPH and hydrogen peroxide radical scavenging activities (57.23 and 39.10%, respectively). The ferrous ion chelating antioxidant power was 82.95% in the water extract of *C. herpestica*, which was the highest value among all the extracts. Generally, phenolic compounds and sulfated polysaccharides are among the major polar components that contribute to antioxidant activity in water-based algae extracts (Fernando et al., 2016a). Moreover, low molecular weight polysaccharides are reported to be effective as scavengers of radicals, whereas unhydrolyzed, longer polysaccharide chains can chelate the Fe^{2+} ions more efficiently than low molecular weight polysaccharides (Jiao et al., 2011).

Toxicity and intracellular ROS scavenging activity

The cytotoxicity of the extracts was determined in Chang human liver cells by using an MTT assay (Fig. 1) after 24-hour incubation with a sample concentration of 100 μg/mL. As indicated in Fig. 1, all extracts, except for the extracts from sample 5 (*C. herpesica*), resulted in a cell viability of over 80%. The cytotoxicity results

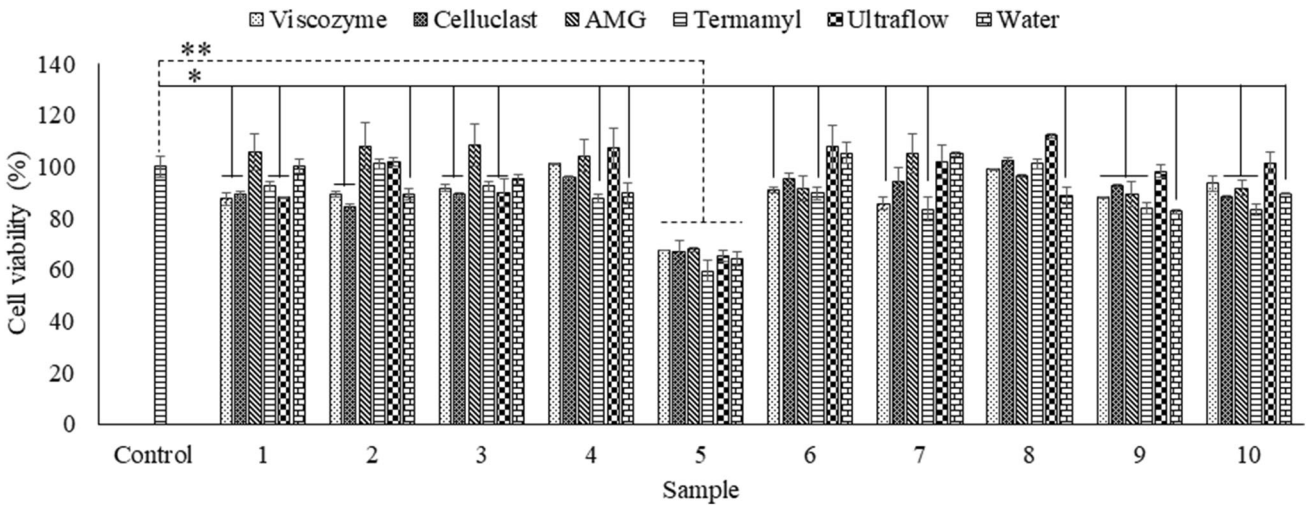

Fig. 1 Cytotoxicity of the algal extracts. The results represent the percentage viability of Chang cells 24 h after the sample treatment. The values were obtained from three independent experiments. *$p < 0.05$, **$p < 0.001$ were considered significant compared with the control

demonstrated that the algal extracts, except for *C. herpestica*, could be safely applied to cells at concentrations lower than 200 μg/mL for further experiments.

Intracellular ROS scavenging abilities of the extracts were evaluated by using the well-known DCFH-DA assay (Table 1). DCFH-DA is easily absorbed into cells and converted into non-fluorescent DCFH by cellular esterases. After oxidation by intracellular ROS and other peroxides, DCFH is converted into the highly fluorescent DCF, which can be detected by using a fluorimeter (excitation wavelength, 485 nm; emission wavelength, 530 nm) (Rastogi et al., 2010). The Celluclast extract of *S. polycystum* (CSp), *S. natans* and *C. minima* showed the strongest intracellular ROS scavenging effects. Overall, the Celluclast extracts exhibited better antioxidant properties than other enzymatic extracts and the DW extracts.

Anti-inflammatory activity of the Celluclast extract of *C. minima* on LPS-induced NO production

As shown in Table 2, a distinct reduction in NO production was induced by the Celluclast extract of *C. minima* (CCm) (IC_{50} = 44.47 μg/mL). In addition, all the different carbohydrase extracts of *C. minima* exhibited stronger anti-inflammatory activities than those from other algae. Most of the extracts possessed anti-inflammatory activities with a cell viability over 80%, except for the *C. herpestica* extracts, which induced cytotoxicity in (RAW cell viability < 60% at 25.00 μg/mL). As described in the previous sub-section, *C. herpestica* extracts were toxic to Chang cells. Given the observed cytotoxicity, none of the extracts from *C. herpestica* were suitable for use in further biological assays. These observations indicated that the EAE

Table 1 IC_{50} values of the algal extracts for intracellular hydrogen peroxide scavenging activity

Sample	Viscozyme (μg/ml)	Celluclast (μg/ml)	AMG (μg/ml)	Termamyl (μg/ml)	Ultraflo (μg/ml)	DW (μg/ml)
1	119.81 ± 3.01^{a}	86.19 ± 4.92^{a}	115.27 ± 2.99^{a}	114.21 ± 5.59^{a}	119.65 ± 0.09^{a}	167.14 ± 3.81^{b}
2	124.06 ± 3.84^{a}	99.65 ± 3.55^{b}	127.99 ± 6.69^{b}	127.95 ± 6.20^{b}	125.45 ± 0.75^{b}	176.05 ± 3.28^{b}
3	136.41 ± 6.44^{b}	125.27 ± 2.88^{c}	137.99 ± 3.03^{b}	138.86 ± 6.15^{c}	130.38 ± 3.62^{c}	186.42 ± 5.87^{c}
4	119.79 ± 0.77^{a}	98.07 ± 4.43^{b}	116.35 ± 2.20^{a}	124.21 ± 3.06^{a}	115.20 ± 2.61^{a}	152.99 ± 0.88^{a}
5	> 200	> 200	> 200	> 200	> 200	> 200
6	156.02 ± 1.99^{c}	148.51 ± 4.31^{d}	153.64 ± 0.66^{c}	160.32 ± 3.05^{d}	164.54 ± 6.14^{d}	190.18 ± 2.44^{c}
7	> 200	> 200	> 200	> 200	> 200	> 200
8	159.49 ± 5.24^{c}	130.33 ± 1.61^{c}	157.12 ± 4.99^{c}	160.54 ± 1.45^{d}	164.17 ± 3.47^{d}	> 200
9	> 200	> 200	> 200	> 200	> 200	> 200
10	184.47 ± 6.70^{d}	162.40 ± 0.23^{e}	181.66 ± 7.00^{d}	173.94 ± 0.43^{e}	179.84 ± 7.06^{e}	> 200

The numbers indicate; 1. *S. polycystum*; 2. *S. natans*; 3. *P. commersonii*; 4. *C. minima*; 5. *C. herpestica*; 6. *C. antennina*; 7. *U. fasciata*; 8. *A. pygmaea*; 9. *G. corticata* and 10. *G. lithophila*. Values are expressed as mean ± standard deviation of triplicate determinations

Values within a column indicated by different letters are significantly different ($p < 0.05$)

Table 2 IC_{50} values of the algal extracts against LPS induced NO production in RAW 264.7 cells as a measurement of anti-inflammatory activity

Sample	Viscozyme (μg/ml)	Celluclast (μg/ml)	AMG (μg/ml)	Termamyl (μg/ml)	Ultraflo (μg/ml)	DW (μg/ml)
1	53.74 ± 2.18[a]	50.28 ± 2.55[a]	63.80 ± 0.46[b]	74.30 ± 1.61[c]	73.49 ± 3.65[b]	86.61 ± 2.87[b]
2	83.28 ± 1.85[c]	58.81 ± 3.35[b]	78.45 ± 2.15[d]	71.24 ± 3.36[c]	77.44 ± 1.56[c]	129.14 ± 0.89[c]
3	80.34 ± 4.03[c]	66.00 ± 0.18[c]	71.10 ± 0.46[c]	64.71 ± 1.37[b]	69.94 ± 2.10[b]	128.27 ± 1.38[c]
4	50.97 ± 3.87[a]	44.47 ± 0.99[a]	59.38 ± 1.77[a]	54.82 ± 0.61[a]	55.07 ± 2.61[a]	66.56 ± 0.96[a]
5	129.99 ± 3.21[g]	124.96 ± 1.61[g]	113.09 ± 0.41[g]	156.95 ± 1.64[f]	109.79 ± 1.58[e]	> 200
6	60.40 ± 0.36[b]	49.55 ± 4.21[a]	65.90 ± 2.35[b]	53.63 ± 2.00[a]	54.63 ± 3.32[a]	148.13 ± 3.08[d]
7	> 200	> 200	> 200	> 200	> 200	> 200
8	99.19 ± 2.15[d]	116.59 ± 4.09[f]	83.61 ± 2.77[e]	60.27 ± 2.81[b]	99.98 ± 2.64[d]	> 200
9	108.87 ± 1.59[e]	109.82 ± 3.88[e]	103.83 ± 0.10[f]	117.23 ± 1.41[e]	103.22 ± 2.94[d]	131.35 ± 0.42[c]
10	119.41 ± 1.45[f]	102.12 ± 2.50[d]	111.66 ± 2.91[g]	111.54 ± 4.17[d]	138.34 ± 2.17[f]	164.44 ± 2.55[e]

The numbers indicate; 1. *S. polycystum*; 2. *S. natans*; 3. *P. commersonii*; 4. *C. minima*; 5. *C. herpestica*; 6. *C. antennina*; 7. *U. fasciata*; 8. *A. pygmaea*; 9. *G. corticata* and 10. *G. lithophila*. Values are expressed as mean ± standard deviation of triplicate determinations

Values within a column indicated by different letters are significantly different ($p < 0.05$)

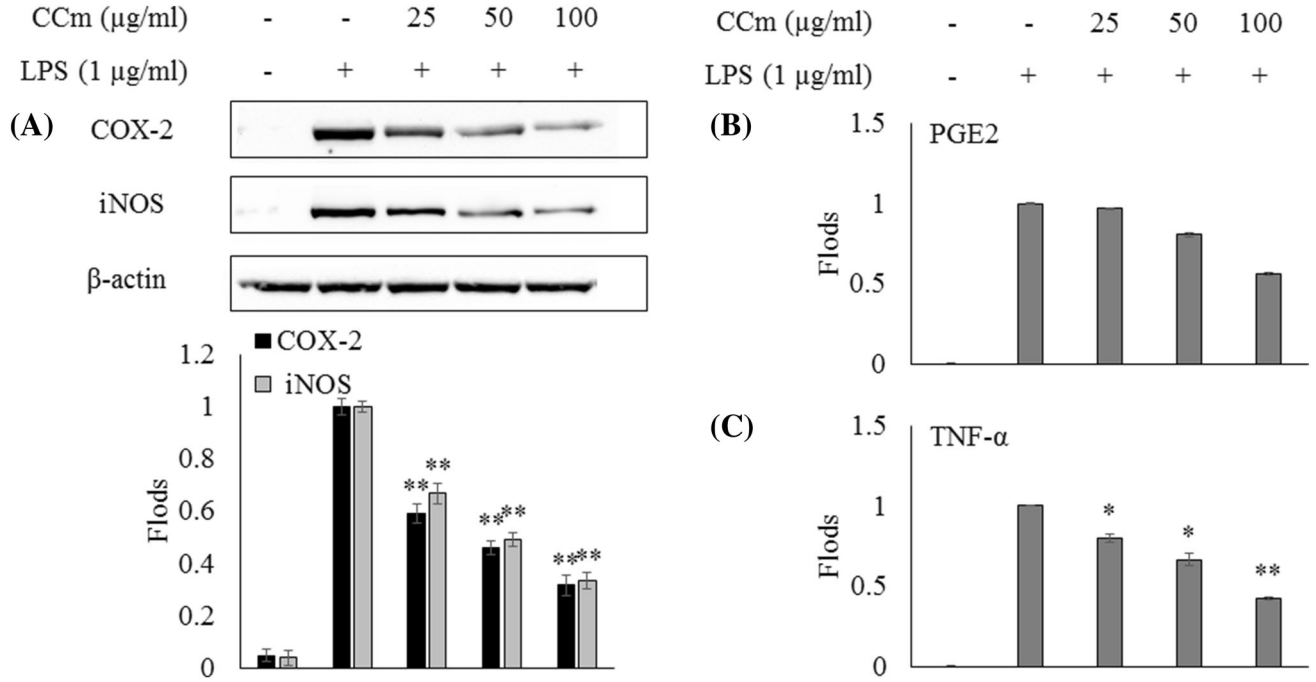

Fig. 2 The effects of the CCm on LPS-induced iNOS and COX-2 protein expression and pro-inflammatory cytokine production in RAW 264.7 cells. Expression analysis of (**A**) iNOS and COX-2 levels using western blot, (**B**) PGE_2 and (**C**) TNF-α levels using Elisa kits. The results were obtained from three independent experiments and presented as the means. The error bars indicate the standard deviations. $*p < 0.05$, $**p < 0.001$ were considered significant compared with the control

of algae achieved a higher extraction yield with better functional properties.

Effect of CCm upon LPS-induced iNOS, COX-2, PGE_2 and TNF-α protein expression in LPS-induced RAW cells

Inflammatory responses are mediated by a complex system of signaling pathways. iNOS, COX-2, PGE_2, and TNF-α are some of the key cell signaling mediators that regulate the production of NO and prostaglandins, which, in turn, regulate an array of cellular responses (Sanjeewa et al., 2016). As shown in Fig. 2, LPS treatment significantly increased cytokine production compared with the control. However, the treatment of CCm effectively suppressed the expression levels of iNOS, COX-2, PGE_2, and TNF-α in a dose-dependent manner. Further, the observed reduction of NO production in RAW 264.7 macrophages could be

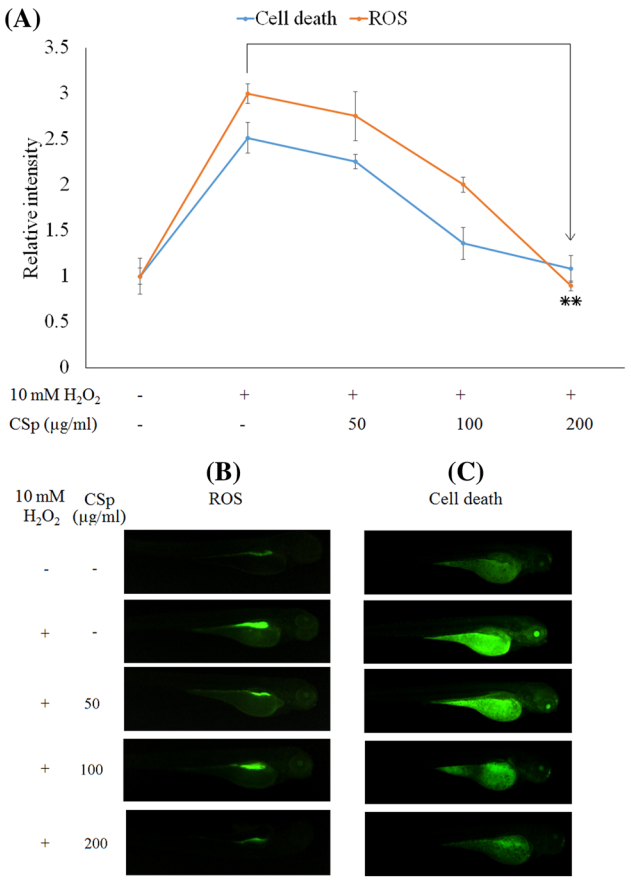

Fig. 3 The in vivo evaluation of the protective effect of CSp against hydrogen peroxide-induced oxidative stress and cell death in zebrafish. (**A**) Relative fluorescence intensities of ROS levels and cell death. (**B**) Microscopic fluorescence images of ROS levels in zebrafish larvae (stained with DCF-DA). (**C**) Microscopic fluorescence images of cell death in zebrafish larvae (stained with acridine orange). At 3 hpf, embryos were mounted in embryo media containing 1.00 mL of 0.2 mM PTU. After 1 h embryos were treated with 50, 100, and 200 μg/mL of CSp. After a further 1 h, 10 μg/mL H_2O_2 was introduced to the embryos. At 3 dpf, the zebrafish larvae were examined by using fluorescence staining methods. The results were obtained from three independent experiments. $*p < 0.05$, $**p < 0.001$ were considered significant compared with the control

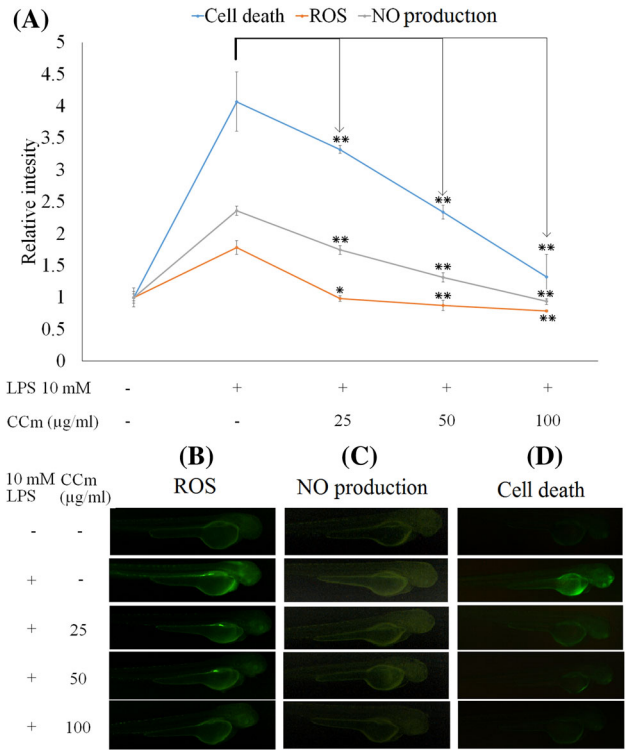

Fig. 4 In vivo evaluation of the protective effect of CCm against LPS-induced NO production, oxidative stress and cell death in zebrafish. (**A**) Relative fluorescence intensities of ROS levels NO production and cell death. (**B**) Microscopic fluorescence images of ROS levels in zebrafish larvae (stained with DCF-DA). (**C**) Microscopic fluorescence images of NO levels in zebrafish larvae (stained with DAF-FM-DA). (**D**) Microscopic fluorescence images of cell death in zebrafish larvae (stained with acridine orange). At 3 hpf, the embryos were mounted in embryo media containing 1.00 mL of 0.2 mM PTU. After 1 h, embryos were treated with 25, 50, and 100 μg/mL of CCm. After a further 1 h, 10 μg/mL LPS was introduced to the embryos. At 3 dpf the zebrafish larvae were examined by using fluorescence staining methods. The results were obtained from three independent experiments. $*p < 0.05$, $**p < 0.001$ were considered significant compared with the control

attributed to the downregulation of iNOS expression. The Celluclast extracts of all algae had higher anti-inflammatory activity than the DW extract, as EAE is a more efficient DW extraction to obtain bioactives from algae. The suppression of the expression of iNOS, COX-2, PGE_2 and proinflammatory cytokines, TNF-α, IL-β, and IL-6, demonstrate the ability of CCm to reduce detrimental inflammatory responses.

Protective effect of CSp against LPS-induced oxidative stress and cell death in zebrafish

The survival rate for the zebrafish treated with 10 mM H_2O_2 at 5 dpf was 58.6%, whereas the treatment with CSp (200 μg/mL) increased survival up to 93.0%. The heart beating rate at 2 dpf was increased to 112.2% in zebrafish treated with 10 mM H_2O_2, with an average of 160 beats/min. The treatment with CSp (200 μg/mL) reduced the rate to the normal levels (100%), with an average of 143 beats/min. Further, the cell death of zebrafish induced by oxidative stress was observed by using acridine orange staining (Fig. 3). The decrease in fluorescence intensities after the treatment with CSp (200 μg/mL) effectively reduced the cell death caused by H_2O_2-induced oxidative stress to normal levels. Intracellular ROS levels were simultaneously analyzed by using the DCFH-DA staining method (Fig. 3). Again, CSp (200 μg/mL) effectively reduced the intracellular ROS levels to those similar to the control. These results provided convincing evidence of the

efficacy of these extracts in animal models, suggesting their possible use in humans.

Anti-inflammatory activity of CCm against LPS-induced NO production, oxidative stress and cell death in zebrafish

The heart rate was markedly increased after LPS treatment in zebrafish, by up to 113.4% (161 beats/min) of the control value (140 beats/min). CCm (100 μg/mL) treatment effectively reduced it to the normal level (142 beats/min). The survival rate of the LPS-treated zebrafish at 5 dpf was 51.7%, but the treatment of CCm at 25, 50, and 100 μg/mL successfully increased the survival rate up to 59.2, 76.5, and 93.1%, which represented a dose-dependent response. As shown in Fig. 4, LPS treatment increased cell death, ROS production, and NO production in zebrafish at 3 dpf, which was effectively ameliorated by 100 μg/mL CCm and returned to levels similar to the control sample. The high fluorescence intensity of the LPS-treated positive control group of zebrafish indicated increased NO production. Treatment with CCm effectively reduced the NO levels, demonstrating the anti-inflammatory effects.

In summary, EAE is a safe and inexpensive method to obtain bioactives from plant material compared with conventional methods. The enzymatic extracts of the ten different Sri Lankan marine algae yielded a higher polyphenolic content with notable antioxidant and anti-inflammatory activities in the Celluclast extracts of *S. polycystum* and *C. minima* both in in vitro and in vivo zebrafish model systems. These results suggested that enzymatic extraction using Celluclast was an efficient method of obtaining bioactives from algae. Further, this study showed the potential of EAE in the industrial manufacture of algae-based functional ingredients.

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